REMARKS

Discussion of Claim Amendments

Claim 39 has been amended to recite n = 1 to 15. In claims 39 - 40, the reference to the keto substituent of alkyl and aryl (in W) has been removed; "aryl heterocyclyl" has been further clarified as one wherein an aryl group is linked to a heterocyclyl group; the proviso relating to Z has been further refined. "Keto" substituent in claim 49 has been removed. Claim 116 has been amended to further clarify arylheterocyclyl. Claim 121 has been amended by replacing "pharmacologically" with -- pharmaceutically --. Claim 136 has been amended to indicate that the treatment is cancer treatment. New claims 137 - 139 have been added and are directed to embodiments of the invention.

No new matter has been added.

The Present Invention

With the entry of the amendment, claims 39-49, 67-68, 73, 78, 85, 86, 91-93, 107, 113, 116-118, and 120-139 would be pending. Claims 86, 91, 92, 93, 107, 113, 118, 122-125, and 131-136 remain withdrawn and are directed to the use of the compounds of the presently claimed invention. A previous Office Action indicated that use claims would be rejoined with composition claims. Accordingly, applicants respectfully request that the above use claims should be rejoined with the composition claims.

The Office Action

The Office Action sets forth the following grounds for rejection: (1) claim 39 is rejected under the doctrine of obviousness-type double patenting as allegedly unpatentable over claim 1, 10, or 11 of Burke (U.S. Patent No. 6,307,090); (2) claims 133 and 136 rejected under 35 U.S.C. § 112, first paragraph, for an alleged non-enablement; (3) claims 39-49, 67, 68, 73, 78, 85, 116, 117, 120-124, 126-130, 133, and 136 are rejected under 35 U.S.C. § 112, second paragraph, for an alleged indefiniteness; (4) claims 39, 40, 49, 72, 78, and 85 are rejected under 35 U.S.C. § 102(a), as allegedly anticipated by Al-Obeidi (USP 5,849,510); (5) claims 39, 40, 49, 67, and 78 are rejected under 35 U.S.C. § 102(e), as allegedly anticipated by Larsen (USP 6,410,585); (6) claims 39, 40, 49, 67, and 78 are rejected under 35 U.S.C. § 102(e), as allegedly anticipated by Horwell (USP 5,981,755); and (7) claim 39 is rejected under 35 U.S.C. § 102(e), as allegedly anticipated by Burke, Hiyoshi (USP 5,824,862), Harding (USP 6,022,696), and Landry (USP 5,948,658).

Discussion of Rejections

1. Obviousness-Type Double Patenting

As claim 39 has been amended, applicants believe that there is no obviousness-type double patenting over claim 1, 10 or 11 of Burke. Claims 1, 10 and 11 of Burke do not suggest to those of ordinary skill in the art the subject matter of claim 39. Particularly, the cited claims do not suggest to those of ordinary skill in the art group Z of the present claim. In view of the foregoing, the obviousness-type double patenting rejection should be removed.

2. Non-Enablement

The Office Action has rejected claims 133 and 136 as allegedly lacking enablement. The Office Action contends that claim 133 is drawn to a method of enhancing the therapeutic effect of an unspecified drug. As therapeutic effect is found in claim 136, the Office Action apparently meant 136 and not 133. Claim 136 clearly recites a compound of claim 120. Thus, the drug is specified. In addition, claim 136 has been amended to recite that the treatment is cancer treatment. As regards the assertion of "unpredictability", applicants respectfully submit that there is sufficient evidence in the literature that Grb2 binding inhibition can lead to cancer treatment. See, e.g., Bardelli et al., *Oncogene* (1999) 18, 1139-1146 (copy enclosed) which discloses that preferential coupling to Grb2 or PI3-kinase

impairs the Tpr-Met metastatic potential; and Tpr-Met mediated metastasis requires concomitant activation of Grb2 and PI 3-kinase-mediated pathways and correlates with invasiveness.

Accordingly, the non-enablement rejection of claims 133 and 136 should be withdrawn. Claims 137-139 are directed to treatment of breast cancer; the Office Action has indicated the possibility of allowing such claims, for which applicants are thankful.

3. Indefiniteness

The Office Action alleges that claims 39, 121, and 136 are indefinite. Applicants have amended these claims, as well as claims 40, 49, and 116. The amended claims meet the statutory requirement. Claims 41-48, 67, 68, 73, 78, 85, 117, 120, 122-124, and 133 are dependent claims. Accordingly, the indefiniteness rejection of claims 39-49, 67, 68, 73, 78, 85, 116, 117, 120-124, 126-130, 133, and 136 should be withdrawn. Claims 137-139 also should not be rejected on this basis.

4. Anticipation

Applicants respectfully traverse the rejection of claims 39, 40, 49, 72, 78, and 85 with respect to Al-Obeidi. Al-Obeidi discloses at column 22, line 15+, a compound wherein the group at the right-hand side (CH_2-pyridyl) is not an arylheterocyclyl alkyl amino. The disclosed compound is merely a heterocyclylalkylamino. Nevertheless, claims 39 and 40 have been amended to further clarify arylheterocyclyl. Claims 49, 78, and 85 are dependent claims. Claim 72 remains canceled. Accordingly, Al-Obeidi fails to anticipate the present claims.

Claims 39, 40, 49, 67 and 78 should be patentable over Larsen as well as Horwell. As discussed, claim 39 has been amended to recite n = 1 - 15. In claim 40 also, n is 1-15. In contrast, Larsen and Horwell disclose compounds wherein n = 0. Claims 49, 67, and 78 are dependent upon claim 39 or 40. Accordingly, claims 39, 40, 49, 67, and 78 should not be rejected.

As claim 39 is now amended (see amended proviso), Burke should not be used to reject this claim. Burke does not disclose the compounds encompassed by amended claim 39.

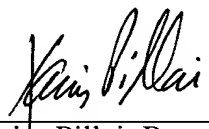
The rejections over Hiyoshi, Harding, and Landry of claim 39 are erroneous. These references disclose compounds wherein Z (in the language of present claims) is phenylethylamino which is substituted with carboxyl and/or with hydroxyl (as in Tyr). The present claims do not encompass such substituted compounds. Z calls for arylalkylamino in claim 39, and not substituted arylalkyl amino. Hiyoshi, Harding, and Landry fail to disclose unsubstituted arylalkylamino.

In view of the foregoing, the anticipation rejections should be removed. Claims 137-139 also should be rejected on the basis.

Conclusion

The application is considered in good and proper form for allowance, and the Examiner is respectfully requested to pass this application to issue. If, in the opinion of the Examiner, a telephone conference would expedite the prosecution of the subject application, the Examiner is invited to call the undersigned attorney.

Respectfully submitted,



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Concomitant activation of pathways downstream of Grb2 and PI 3-kinase is required for *MET*-mediated metastasis

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The *Met* tyrosine kinase – the HGF receptor – induces cell transformation and metastasis when constitutively activated. *Met* signaling is mediated by phosphorylation of two carboxy-terminal tyrosines which act as docking sites for a number of SH2-containing molecules. These include Grb2 and p85 which couple the receptor, respectively, with *Ras* and PI 3-kinase. We previously showed that a *Met* mutant designed to obtain preferential coupling with Grb2 (Met^{2xGrb2}) is permissive for motility, increases transformation, but – surprisingly – is impaired in causing invasion and metastasis. In this work we used *Met* mutants optimized for binding either p85 alone (Met^{2xPI3K}) or p85 and Grb2 ($Met^{PI3K/Grb2}$) to evaluate the relative importance of Ras and PI 3-kinase as downstream effectors of *Met*. Met^{2xPI3K} was competent in eliciting motility, but not transformation, invasion, or metastasis. Conversely, $Met^{PI3K/Grb2}$ induced motility, transformation, invasion and metastasis as efficiently as wild type *Met*. Furthermore, the expression of constitutively active PI 3-kinase in cells transformed by the Met^{2xGrb2} mutant, fully rescued their ability to invade and metastasize. These data point to a central role for PI 3-kinase in *Met*-mediated invasiveness, and indicate that simultaneous activation of *Ras* and PI 3-kinase is required to unleash the *Met* metastatic potential.

Keywords: *Met*-mediated metastasis; *Met* signaling; PI 3-kinase; Grb2; *Ras*

Introduction

Hepatocyte growth factor (HGF), also known as Scatter Factor (SF), is a mesenchymal cytokine which triggers in target cells a unique biological program leading to invasive cell growth (reviewed in Jeffers *et al.*, 1996; Bardelli *et al.*, 1997a). The latter results from the integration of multiple biological responses to HGF such as cell proliferation, survival, motility, and invasion of extracellular matrices (Weidner *et al.*, 1993; Medico *et al.*, 1996; Bardelli *et al.*, 1996; Fan *et al.*, 1998). The HGF receptor is a transmembrane tyrosine kinase encoded by the c-*MET* proto-oncogene (Bottaro *et al.*, 1991; Naldini *et al.*, 1991). c-*MET* is overexpressed and amplified in a number of human tumors (reviewed in Bardelli *et al.*, 1997a). Recently, a direct genetic link between c-*MET* and human cancer was established by the identification of activating mutations in the c-*MET* gene in hereditary papillary renal carcinomas (Schmidt *et al.*, 1997; Fischer *et al.*, 1998; Zhuang *et al.*, 1998). c-*MET* was originally identified as the cellular counterpart of a transforming gene, *TPR–MET*, resulting from a chromosomal rearrangement (Cooper *et al.*, 1984; Park *et al.*, 1986). In *Tpr–Met*, the extracellular domain of *Met* is replaced by *Tpr* sequences, which provide two strong dimerization motifs (Rodrigues and Park, 1993). Dimerization causes constitutive activation of the *Met* kinase, which acquires transforming and metastatic properties (Park *et al.*, 1986; Giordano *et al.*, 1997). *Met* signaling and *Tpr–Met* mediated transformation are based on the activation of multiple pathways triggered by phosphorylation of two carboxy-terminal tyrosines (Y_{1349}VHVNATY$_{1356}$VNV, Ponzetto *et al.*, 1993, 1994; Fixman *et al.*, 1995). These tyrosine residues are part of a consensus sequence (YVH/NV) which mediates coupling of the receptor with several effectors, including the Grb2/SoS complex (Ponzetto *et al.*, 1994; Fixman *et al.*, 1995), the p85 regulatory subunit of PI-3-kinase (Ponzetto *et al.*, 1993), Stat-3 (Boccaccio *et al.*, 1998), and the multiadaptor protein Gab1 (Weidner *et al.*, 1996; Bardelli *et al.*, 1997b; Nguyen *et al.*, 1997). Grb2, in particular, requires an Asn in the +2 position for binding, and is thus linked to the receptor via the Y_{1356}VNV motif (Ponzetto *et al.*, 1996; Maina *et al.*, 1996; Fixman *et al.*, 1997).

In previous work we mutagenized the residues in position +2 of the multifunctional tyrosines to either abrogate (Y_{1349}VHVNATY$_{1356}$V<u>N</u>V→Y_{1349}VHVNAT-Y$_{1356}$V<u>H</u>V, Met^{Grb2-}) or to enhance (Y_{1349}V<u>H</u>VNAT-Y$_{1356}$VNV→Y_{1349}V<u>N</u>VNATY$_{1356}$VNV, Met^{2xGrb2}) direct binding with Grb2 (Ponzetto *et al.*, 1996). The Met^{Grb2-} mutation severely impaired the transforming activity of *Tpr–Met* thus showing that a direct link with the *Ras* pathway is required for *Met*-mediated transformation. Conversely, the introduction of a second Grb2 binding site (Met^{2xGrb2}) enhanced transformation but, surprisingly, impaired metastasis (Giordano *et al.*, 1997). These results suggest that the oncogenic form of the *Met* receptor may require direct coupling with additional downstream effector(s) besides Grb2, to be fully metastatic. Such effector(s) could be displaced by the insertion of a second high affinity binding site for Grb2. As a possible candidate we considered PI 3-kinase, which binds Y_{1349} and Y_{1356} at low affinity (Ponzetto *et al.*, 1993). PI 3-kinase is a

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major mediator of HGF-mediated motility (Royal and Park, 1995; Kwaja *et al.*, 1998), invasion (Keely *et al.*, 1997; Shaw *et al.*, 1997), and prevention from apoptosis (Kauffman-Zeh *et al.*, 1997), all events relevant to the metastatic process.

In this work, the role of PI 3-kinase in *Met*-mediated cell motility, transformation, and metastasis was evaluated in two ways: (i) we tested the response of cells expressing *Met* mutants in which, to preferentially activate PI 3-kinase, either one or both docking sites were converted into the optimal motifs for p85; (ii) we evaluated the effect of introducing a constitutively active catalytic subunit of PI 3-kinase in cells transformed by the poorly metastatic Met^{2xGrb2} mutant.

The results of these studies show that concomitant activation of *Ras* and PI 3-kinase is required to sustain the metastatic phenotype of *Met*-transformed cells, and suggest a central role for PI 3-kinase in the transition of tumor cells to the invasive phenotype.

Results

Construction of Tpr–Met *signaling mutants*

Signaling mutants of *Tpr–Met* (the oncogenic form of the *Met* receptor) were generated by site-directed mutagenesis (Table 1). To preferentially activate PI 3-kinase, two optimal consensus sequences reproducing the p85 binding sites of the PDGF receptor (Fantl *et al.*, 1992; Songyang *et al.*, 1993) were introduced downstream to the tyrosine residues responsible for signal transduction (YVHVNATYVNV→YMPMNATYM-DM: $Tpr–Met^{2xPI3K}$). To enhance binding with PI 3-kinase without uncoupling Grb2, only the first of the two sites was converted into an optimal p85 binding motif: (YVHVNATYVNV→YMPMNATYVNV: $Tpr–Met^{PI3K/Grb2}$).

Also included in the experiments were three previously described mutants designed to specifically uncouple Grb2 (YVHVNATYVNV→YVHVNATYVHV: $Tpr–Met^{Grb2-}$), to enhance Grb2 binding (YVHVNA-→YVNVNATYVNV: $Tpr–Met^{2xGrb2}$) and to abrogate signal transduction ($Tpr–Met^{Double}$: YVHV-NATYVNV→FVHVNATFVNV) (Ponzetto *et al.*, 1996).

Tpr–Met mutants carrying optimized consensus sequences bind and activate Grb2 or PI 3-kinase with increased efficiency

We verified that the new consensus sequences did not interfere with the activity of the *Met* kinase and with phosphorylation of the tyrosine residues using lysates of COS-1 cells expressing the *Tpr–Met* mutants for immunoprecipitation and kinase assay. Figure 1a shows the result of an autokinase assay on the various mutants. In this assay the intensity of the *Tpr–Met* band reflects the level of phosphate incorporation at the major phosphorylation site, Y_{1235}, located in the catalytic domain (Ferracini *et al.*, 1991). The level of auto-kinase activity of all mutants is comparable to wild type, as indicated by the fact that the labeled bands are all of similar intensity (the amounts of *Tpr–Met* protein was comparable in all samples, as assessed by Western blot, data not shown). Differences in phosphorylation of the carboxyterminal tail affect the mobility in SDS–PAGE of the *Tpr–Met* band (Ponzetto *et al.*, 1994). All the *Tpr–Met* mutants appear to be phosphorylated on both tyrosines, as shown by the fact that their migration rate is equivalent to that of $Tpr–Met^{Wt}$. The only exception is $Tpr–Met^{Double}$, which, given the lack of the two carboxyterminal tyrosines, has a higher migration rate.

Table 1 *Tpr–Met* signaling mutants designed to preferentially bind Grb2 or PI 3-kinase

$Tpr–Met^{Wt}$	YVHVNATYVNV
$Tpr–Met^{Double}$	FVHVNATFVNV
$Tpr–Met^{Grb2-}$	YVHVNATYVHV
$Tpr–Met^{2\times Grb2}$	YVNVNATYVNV
$Tpr–Met^{2\times PI3K}$	YMPMNATYMDM
$Tpr–Met^{PI3K/Grb2}$	YMPMNATYVNV

Signaling mutants of the oncogenic form of the *Met* receptor (*Tpr–Met*) were generated by site-directed mutagenesis. The consensus sequences for the SH2 domains of Grb2 and p85 (the regulatory subunit of PI 3-kinase) were designed according to Songyang *et al.* (1993). Mutagenized residues are underlined

Transfected COS-1 cells were also used to assess the relative ability of the *Tpr–Met* signaling mutants to bind Grb2 or PI 3-kinase. For Grb2, lysates of COS-1 cells expressing the *Tpr–Met* mutants were incubated with GST-Grb2 fusion protein immobilized on Glutathione-Sepharose beads, and bound *Tpr–Met* was visualized by autokinase assay. Figure 1b shows that Grb2 binds a higher amount of $Tpr–Met^{2xGrb2}$ compared to $Tpr–Met^{Wt}$ and to $Tpr–Met^{PI3K/Grb2}$. The $Tpr–Met^{2xPI3K}$ mutant, $Tpr–Met^{Grb2-}$, as well as $Tpr–Met^{Double}$ do not associate Grb2. In agreement with these associations data we have shown that $Tpr–Met^{2xGrb2}$ elicits an increased response with respect to $Tpr–Met^{Wt}$ from a *Ras*-responsive promoter, while the ability of $Tpr–Met^{2xPI3K}$ and of $Tpr–Met^{Grb2-}$ to elicit a *Ras*-mediated response is impaired (Besser *et al.*, 1997). For PI 3-kinase, lysates of COS-1 cells transfected with the various *Tpr–Met* mutants were immunoprecipitated with anti-*Met* antibodies and blotted with antibodies specific for p85. Figure 1c shows that the mutants with optimal p85 binding site(s) ($Tpr–Met^{2xPI3K}$ and $Tpr–Met^{PI3K/Grb2}$) co-precipitate with an amount of p85 strikingly higher compared to wild type, which is undetectable at this level of exposure. Accordingly, the PI 3-kinase activity associated with $Tpr–Met^{2xPI3K}$ or $Tpr–Met^{PI3K/Grb2}$ is higher in lysates of cells expressing these mutants, compared to that found in cells expressing $Tpr–Met^{Wt}$ (Graziani *et al.*, 1991) or $Tpr–Met^{2xGrb2}$ (Figure 1d). The presence of one or two optimal p85 binding site(s) ($Tpr–Met^{PI3K/Grb2}$, $Tpr–Met^{2xPI3K}$) does not seem to affect the amount of co-immunoprecipitated p85 or PI 3-kinase activity. This may indicate that in COS cells the level of overexpressed *Tpr–Met* protein exceeds the amount of p85-p110 available for binding.

Enhanced coupling of Met to PI 3-kinase elicits motility in epithelial cells

The effects of the signaling mutations on *Met*-mediated motility were tested in MDCK cells, an epithelial cell line which grows in culture in tight islands and responds to HGF by 'scattering' (Stoker *et al.*, 1987). MDCK cells were co-transfected with the different *Tpr–Met* constructs and the plasmid psV2neo. G418

resistant clones were picked and expanded. Expression of *Tpr–Met* was determined by either immunoprecipitation and kinase assay, or by Western blot. Clones expressing each of the *Tpr–Met* constructs, with the exception of *Tpr–Met*^{Double}, had a constitutively motile fibroblast-like phenotype (Figure 2a). The clones shown in Figure 2a expressed comparable amounts of *Tpr–Met* proteins (Figure 2b). Thus, preferential coupling with Grb2 or PI 3-kinase is sufficient to elicit the *Met*-mediated motility response.

Preferential coupling to Grb2 on PI 3-kinase has opposite effects on Tpr–Met*-mediated transformation*

The transforming potential of the *Tpr–Met*^{2xPI3K} and *Tpr–Met*$^{PI3K/Grb2}$ mutants was evaluated in a focus forming assay, using Rat Fisher fibroblasts. We have previously shown that the mutation abrogating the link with Grb2 (*Tpr–Met*$^{Grb2-}$) severely impairs transformation, and that duplication of the Grb2 binding site (*Tpr–Met*^{2xGrb2}) enhances transformation (Ponzetto *et al.*, 1996). Figure 3 shows that preferential coupling of *Tpr–Met* with PI 3-kinase (*Tpr–Met*^{2xPI3K}) drastically lowers the numbers of foci compared to the wild type control, indicating that activation of PI 3-kinase is not sufficient for efficient transformation. On the other hand, the *Tpr–Met*$^{PI3K/Grb2}$ mutant, modified to preferentially activate PI 3-kinase together with *Ras*, is fully competent in mediating transformation (Figure 3). These results indicate that while the requirement for motility can be fulfiled by either one of the two pathways, the *Met*-mediated proliferative signal requires a threshold of *Ras* activation and cannot be sustained by PI 3-kinase alone.

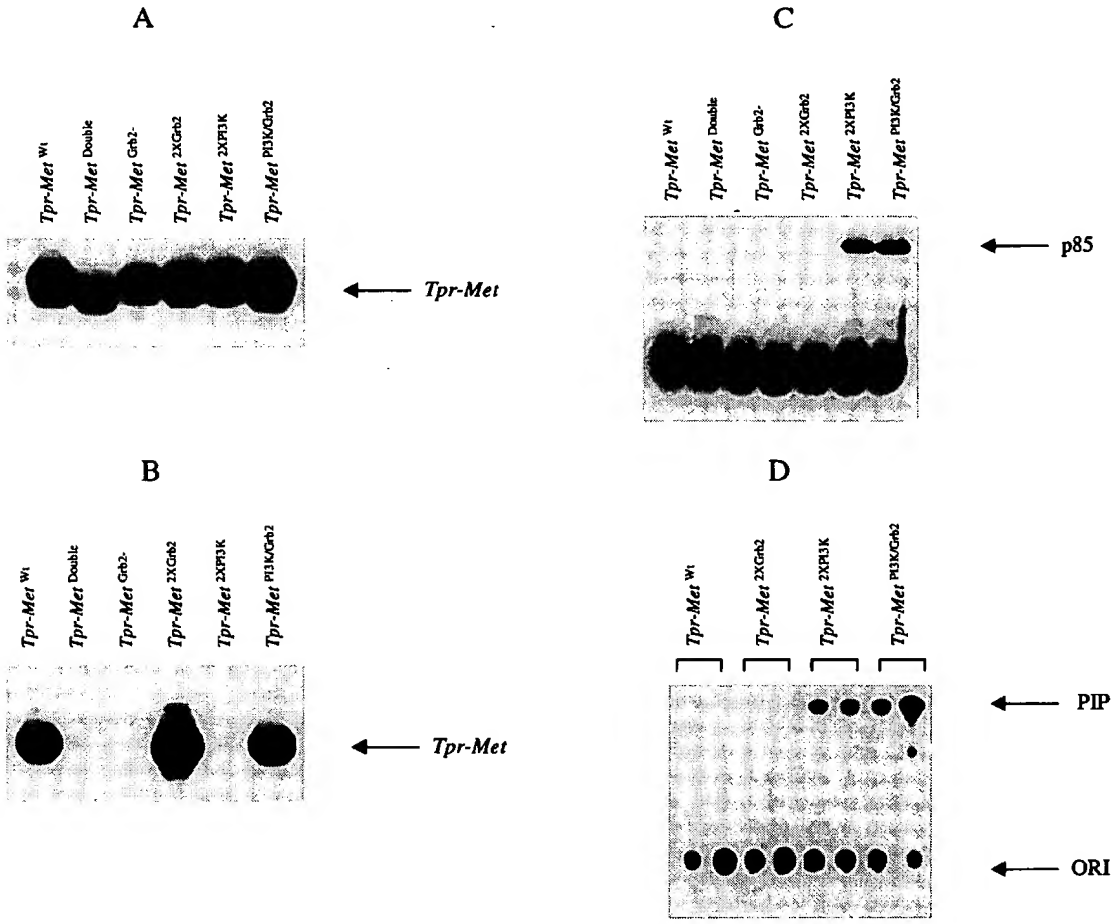

Figure 1 Kinase activity and association of *Tpr–Met* signaling mutants with Grb2 and PI 3-kinase. (a) Wild type and mutant *Tpr–Met* proteins were immuno-precipitated from COS-1 cells transfected with the corresponding constructs, using antibodies specific for human *Met* and subjected to *in vitro* kinase assay with [γ-^{32}P]ATP. Labeled proteins were separated on 8% SDS–PAGE. The gel was dried and exposed for autoradiography. (b) Grb2 fusion protein (approximately 500 ng/point) was immobilized on Glutathione-Sepharose beads and incubated with lysates of COS-1 cells containing comparable amounts of *Tpr–Met* mutants. Complexes were washed and the amount of *Tpr–Met* bound to Grb2 was visualized by *in vitro* kinase assay with [γ-^{32}P]ATP. Labeled proteins were separated on 8% SDS–PAGE. The gel was dried and exposed for autoradiography. (c) *Tpr–Met* mutants were immunoprecipitated with anti-*Met* antibodies from lysates of transfected COS-1 cells. Immunoprecipitates were separated on 8% SDS–PAGE. Associated p85 was visualized by Western blotting with anti-p85 antibodies. Exposure was optimized to show the amount of p85 associated to the *Tpr–Met*^{2xPI3K} and *Tpr–Met*$^{PI3K/Grb2}$ mutants. Upon longer exposure a faint p85 band can also be detected in the *Tpr–Met*^{Wt} lane, as previously shown (Graziani *et al.*, 1991). (d) *Tpr–Met* mutants were immunoprecipitated with anti-Met antibodies from lysates of transfected COS-1 cells and the amount of *Tpr–Met* associated PI 3-kinase activity was determined by PI 3-kinase assay. The position of the phosphatidylinositol-3-phosphate (PIP) product of the PI 3-kinase reaction is indicated

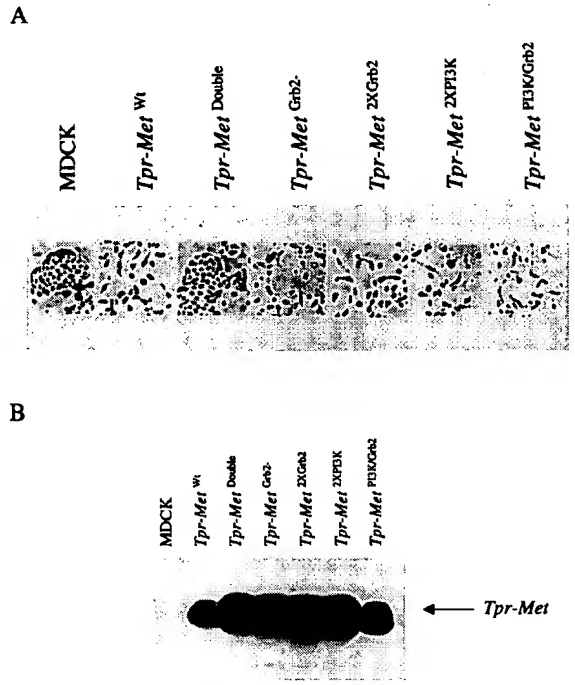

Figure 2 *Tpr–Met* signaling mutants that preferentially bind Grb2 and PI 3-kinase induce constitutive motility in epithelial cells. (a) MDCK cells expressing *Tpr-Met* wild type and *Tpr–Met* signaling mutants show a constitutive motile phenotype with the only exception of those expressing $Tpr–Met^{Double}$. Magnification: 10×. (b) Levels of expression of *Tpr–Met* protein in the MDCK cells shown in panel a. Lysates of MDCK cells expressing wild type *Tpr–Met* and *Tpr–Met* signaling mutants were immunoprecipitated with anti-*Met* antibodies, separated on 8% SDS–PAGE, blotted, and probed with anti-*Met* antibodies

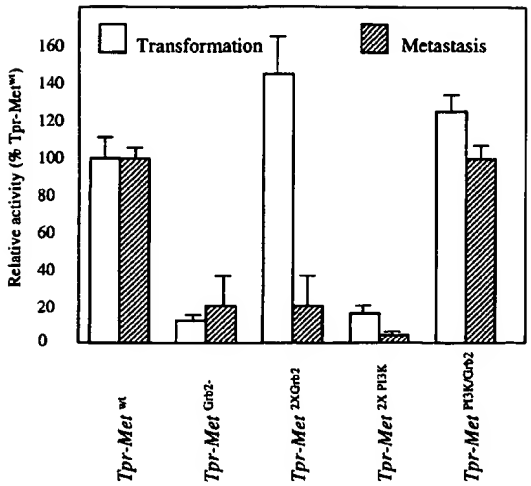

Figure 3 Preferential coupling to Grb2 or PI 3-kinase impairs the metastatic potential of *Tpr–Met*. The transforming potential of the indicated *Tpr–Met* mutants was evaluated by focus forming assay using Fisher rat fibroblasts. The values reported are expressed as percentage of the $Tpr–Met^{Wt}$ transforming activity (150±12 foci/10 μg of DNA) and represent the average of three independent experiments. The metastatic ability of *Tpr–Met* signaling mutants was tested in an experimental metastasis assay (see Materials and methods for details). Bars indicate the percentage of mice dead because of lung metastases 2 weeks after injection into the tail vein of cells transformed by the various *Tpr–Met* signaling mutants. The values reported are expressed as percentage of the lethality caused by $Tpr–Met^{Wt}$ and represent the average of three independent experiments (18 mice/group)

Preferential coupling to Grb2 or PI 3-kinase impairs the Tpr–Met *metastatic potential*

Fibroblasts transformed by *Tpr–Met* signaling mutants were tested in an experimental metastasis assay. Pools of cells deriving from foci of transformation were injected into the tail vein of nude mice. All the animals injected with cells transformed by $Tpr–Met^{Wt}$ died within a short latency time (2 weeks), with massive metastatic colonization of the lungs. Cells transformed by either $Tpr–Met^{2xGrb2}$ or $Tpr–Met^{2xPI3K}$ were severely impaired in their metastatic potential (Figure 3). This indicates that preferential coupling of *Tpr–Met* with either *Ras* or PI 3-kinase interferes with its ability to induce metastasis. Interestingly, the *Tpr–Met* mutant capable of activating both Ras and PI 3-kinase ($Tpr–Met^{PI3K/Grb2}$) was transforming and metastatic. These data suggest that, while coupling with Grb2 is required and sufficient for *Tpr–Met*-mediated transformation, to induce metastasis the *MET* oncogene may need to concomitantly activate PI 3-kinase.

Tpr–Met *mediated metastasis requires concomitant activation of Grb2 and PI 3-kinase-mediated pathways and correlates with invasiveness*

To establish whether activation of PI 3-kinase could rescue to a fully metastatic phenotype cells transformed by $Tpr–Met^{2xGrb2}$, we used a constitutively activated variant of the PI 3-kinase catalytic subunit (p110*). p110* is activated *via* addition of a carboxy-terminal farnesylation signal which relocates the enzyme to the plasma membrane (Khwaja *et al.*, 1997). Cells transformed by the poorly metastatic $Tpr–Met^{2xGrb2}$ and $Tpr–Met^{Grb2-}$ mutants were co-transfected with a plasmid encoding p110* and psV2neo. G418-resistant clones were pooled and assayed for p110* expression by Western blot. Pools of cells expressing comparable amounts of p110* (Figure 4a) were tested in an experimental metastasis assay. Expression of constitutively active PI 3-kinase conferred to $Tpr–Met^{2xGrb2}$ transformed cells (high *Ras* signal, Besser *et al.*, 1997) a strongly metastatic phenotype, which had no effect on cells transformed by $Tpr–Met^{Grb2-}$ (low *Ras* signal; Besser *et al.*, 1997) (Figure 4b). These results indicate that concomitant activation of *Ras* and PI 3-kinase unmasks the metastatic potential of the *MET* oncogene. PI 3-kinase has been shown to play a role in invasion (Shaw *et al.*, 1997). We therefore evaluated whether there was a correlation between the metastatic potential of *Tpr–Met* transformed cells and the acquisition of the ability to cross a barrier of extracellular matrix. Cells expressing $Tpr–Met^{Wt}$, $Tpr–Met^{PI3K/Grb2}$, $Tpr–Met^{Grb2-/p110*}$ or $Tpr–Met^{2xGrb2/p110*}$ were tested in an invasion assay using Matrigel-coated Transwells. Metastatic cells showed increased invasiveness with respect to non-metastatic controls (Figure 4c).

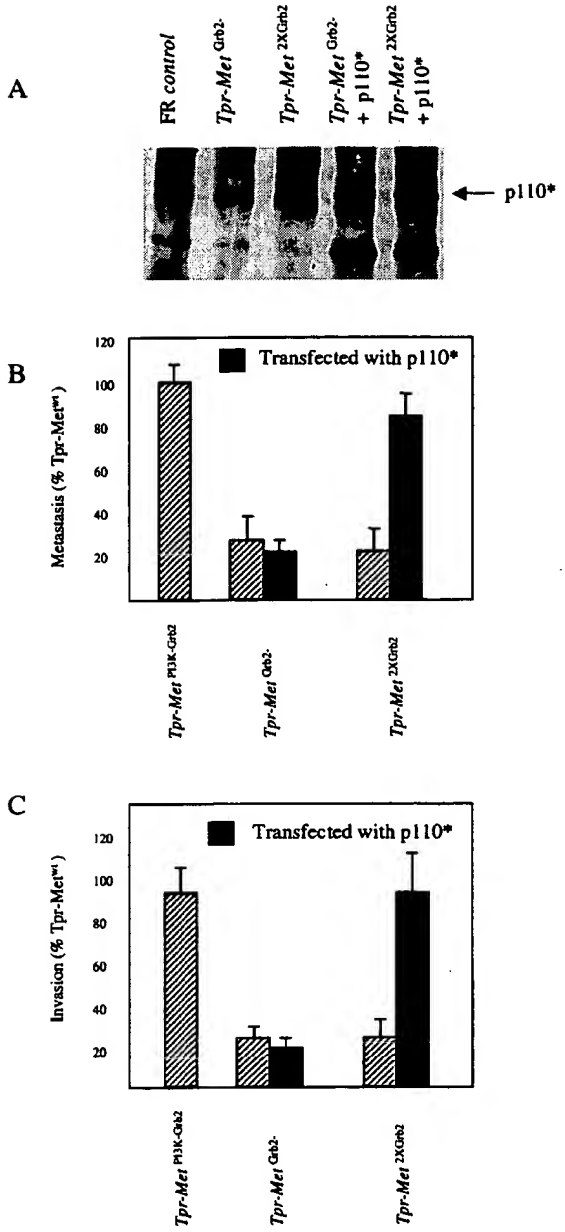

Figure 4 Concomitant activation of Grb2 and PI 3-kinase is required for *Tpr–Met*-mediated metastasis. (**a**) Levels of expression of the catalytic subunit of PI 3-kinase (p110*, Myc tagged; Khwaja *et al.*, 1997) in Fisher rat fibroblasts transformed by the various *Tpr–Met* mutants. Cell lysates were immunoprecipitated with anti-Myc antibodies, separated on 8% SDS–PAGE, blotted, and probed with anti-Myc antibodies. (**b**) The metastatic ability of cells expressing the indicated *Tpr–Met* mutants either alone or together with the constitutively active variant of the PI 3-kinase catalytic subunit (p110*) was evaluated in an experimental metastasis assy. Bars indicate the percentage of mice dead because of lung metastases, two weeks after injection of cells transformed by the various *Tpr–Met* mutants into the tail vein. The values reported are expressed as percentage of the *Tpr–Met*^{Wt} lethality and represent the average of three independent experiments (18 mice/group). (**c**) The invasive potential of cells expressing the indicated *Tpr–Met* mutants alone or together with the constitutively active variant of the PI 3-kinase catlytic subunit (p100*) was evaluated by Transwell invasion assay. Fibroblasts were plated on the upper side of Matrigel-coated Transwell chambers and incubated for 48 h. The values reported are expressed as percentage of the *Tpr–Met*^{Wt} invasive potential and represent the number of cells that had dived into the lower compartment of the Transwell. Values are the averages of three independent experiments performed in duplicate

Discussion

Acquisition of a metastatic phenotype by cancer cells is considered a key step in tumor progression. This process has been shown to correlate with alteration of a number of biological properties among which are cell growth, motility, secretion of proteases, and invasion of extra-cellular matrices (reviewed in Sporn, 1997). The *Met* ligand, HGF/SF, has been shown to influence all of these parameters in target cells (reviewed in Jeffers *et al.*, 1996; Bardelli *et al.*, 1997a). Accordingly, the constitutively active oncogenic counterpart of the *Met* receptor (*Tpr–Met*) transforms fibroblasts at high efficiency, and renders them highly metastatic (Park *et al.*, 1986; Giordano *et al.*, 1997). *Met* signaling and transformation depend on ligand-induced phosphorylation of two carboxy-terminal docking sites of mixed specificity (Y_{1349}/VHVNATY$_{1356}$/VNV, Ponzetto *et al.*, 1994; Fixman *et al.*, 1995; Maina *et al.*, 1996). These phosphotyrosines are responsible for recruiting a number of SH2-containing effectors, including p85 (the regulatory subunit of PI 3-kinase) and the Grb2 adaptor (Ponzetto *et al.*, 1994). While p85 binds at low affinity to either site, Grb2 binds at high affinity to Y_{1356}VNV, and links the receptor directly to the *Ras* pathway via the Grb2/SoS complex (Ponzetto *et al.*, 1993, 1994; Fixman *et al.*, 1997). In previous work we duplicated the endogenous Grb2 binding site (Y_{1349}VHVNATY$_{1356}$VNV → Y_{1349}VNVNATY$_{1356}$VNV) to enhance *Tpr–Met*-mediated *Ras* signaling. This mutant caused an increased response from a *Ras*-responsive promoter (Besser *et al.*, 1997) and was more transforming than wild type (Ponzetto *et al.*, 1996). However, cells transformed by this mutant were impaired in their ability to invade extra-cellular matrices *in vitro* and to induce metastasis in nude mice (Giordano *et al.*, 1997).

In this paper, to investigate the role of PI 3-kinase in *Met*-mediated events (motility, transformation and metastasis), we used the same approach of 'optimizing' the receptor docking sites to bind a specific effector. The new mutants were engineered to preferentially activate PI 3-kinase either alone (*Tpr–Met*^{2xPI3K}), or in combination with *Ras* (*Tpr–Met*$^{PI3K/Grb2}$). This was done by modifying the three residues downstream to one or both of the multifunctional tyrosines into optimal p85 consensus sequences (YMPM, YMDM: Songyang *et al.*, 1993). It should be noted, however, that according to Larose *et al.* (1995) the p85 consensus pYMXM is also compatible with PLC-γ binding, unless a Ser residue is inserted in position +4. In our mutants the residues in position +4 are Asn and Lys. Thus the two new mutants, in addition to having acquired a much stronger PI 3-kinase binding site than *Tpr–Met*^{Wt}, might retain the ability of coupling with PLC-γ. The mutations were inserted in the constitutive active oncogenic form of the receptor *Tpr–Met*, and the constructs were transfected in epithelial cells (MDCK) or in rat Fisher fibroblasts to study the effect of the mutations, respectively, on motility and transformation/metastasis.

All mutants imparted a constitutively 'scattered' phenotype to MDCK cells. In particular, the effect of *Tpr–Met*^{2xPI3K} which selectively binds p85 (Y_{1349}MPMNATY$_{1356}$YMDM), was undistinguishable from that of *Tpr–Met*^{Wt}, suggesting that activation of

PI 3-kinase is sufficient to induce scattering. HGF/SF-induced *Ras* and PI 3-kinase activation have been shown to be both necessary for the scattering response, using dominant negative constructs or specific inhibitors (Hartmann *et al.*, 1994; Derman *et al.*, 1996). Recently, expression of constitutively activated PI 3-kinase has been shown to be sufficient to cause scattering in MDCK cells, provided that a basal level of MAP kinase activity is present (Khwaja *et al.*, 1998). We have previously shown that the *Tpr–Met*^{2xPI3K} mutant elicits from a *Ras* responsive promoter a response which is 25% of wild type (Besser *et al.*, 1997). This threshold of *Ras* signaling combined with *Met*-mediated PI 3-kinase activation is thus sufficient to confer motility to epithelial cells. Conversely, the fact that the *Tpr–Met*^{2xPI3K} mutant was inefficient in a focus forming assay indicates that a higher threshold of *Met*-mediated *Ras* activation is required for transformation.

When cells transformed by the *Tpr–Met*^{2xPI3K} mutant were tested in an experimental metastasis assay, they were impaired in causing lung metastastes in nude mice, behaving like those transformed by the mutant optimized for Grb2 binding (*Tpr–Met*^{2xGrb2}: Y_{1349}VNVNATY$_{1356}$VNV). Thus, by tilting the balance of downstream signaling toward preferential activation of one of the two pathways we interfere with the ability *Tpr–Met* to induce a metastatic phenotype.

Furthermore, a fully metastatic *Tpr–Met* mutant was obtained by mutating the degenerate Y_{1349} VHV site into an optimal motif for PI 3-kinase, while leaving unmodified the endogenous Grb2 binding site (*Tpr–Met*$^{PI3K/Grb2}$: Y_{1349}MPMNATY$_{1356}$VNV). This suggested that it is sufficient to activate PI 3-kinase concomitantly with Grb2-dependent pathways to elicit the invasive/metastatic response. To verify this hypothesis directly, cells transformed by the poorly metastatic *Tpr–Met*^{2xGrb2} mutant were transfected with a constitutively active form of the catalytic subunit of PI 3-kinase (p110*; Khwaja *et al.*, 1997). Expression of p110* fully rescued the metastatic ability of these cells, confirming that both pathways are required for metastasis.

The results of our studies with the Grb2- or PI 3-kinase-directed *Met* signaling mutants highlight the synergistic effect the combined two pathways have in mediating the complex series of events leading to the acquisition of a fully metastatic phenotype. It seems likely that the picture of *Ras* and PI 3-kinase cooperation emerging from the use of these mutants could, by and large, reflect how the wild type version of *Met* naturally implements the biological effects of HGF/SF. While there is clear evidence that PI 3-kinase is a *Met* downstream target (Graziani *et al.*, 1991; Royal and Park, 1995; Derman *et al.*, 1996; Khwaja *et al.*, 1998) the process whereby PI 3-kinase is activated by wild type *Met* is not completely understood. The two carboxy-terminal multifunctional tyrosines in the receptor are relatively low affinity binding sites for p85 (Ponzetto *et al.*, 1993; Songyang *et al.*, 1993). Conversely, three 'optimal' p85 binding motifs are present in the multiadaptor GAB-1, a *Met* substrate which binds the receptor via Grb2 (Holgado-Madruga *et al.*, 1996; Weidner *et al.*, 1996; Bardelli *et al.*, 1997b; Fixman *et al.*, 1997). Thus, PI 3-kinase activation *in vivo* is likely to occur mainly indirectly, through Grb2-mediated GAB-1 phosphorylation. If this is the case, insertion of a second Grb2 binding site should not interfere with PI 3-kinase activation. However, the biological defect of the *Tpr–Met*^{2xGrb2} mutant and its rescue by the activated form of PI 3-kinase, indicate that the mutation somehow upsets the balance between the two pathways. In this respect, it is interesting to note the changes that occurred in the multifunctional docking site of c-*Sea* (another member of the *Met* family of receptors, Huff *et al.*, 1993), after the sequence was acquired by the avian erythroblastosis virus (Smith *et al.*, 1989). In c-*Sea* the two tyrosines responsible for signal transduction are both Grb2 binding site (YVNLAVTYVNL). Accordingly, the corresponding *Tpr–Sea* is transforming, but not highly metastatic (Giordano *et al.*, 1997). Interestingly, v-*Sea* acquired a mutation imparting the ability to bind also PI 3-kinase to the first Grb2 binding site (YLNMAVTYVNL). It is highly suggestive that the oncogenic counterpart of the c-*Sea* receptor should be a variant which has acquired the potential for concomitant activation of both pathways.

Regardless of the precise mechanisms through which *Met* achieves PI 3-kinase activation, this work establishes a critical role for this pathway in the acquisition of the metastatic properties of *Met*-transformed cells.

Materials and methods

Reagents, cells and antibodies

All reagents, unless specified, were purchased from Sigma Chemical Co. Protein A covalently coupled to Sepharose was purchased from Pharmacia LKB Biotechnology Inc. Radioactive isotopes were purchased from Amersham Corp. MDCK cells, COS-1 cells and Fisher rat fibroblasts were purchased from ATCC (American Type Culture Collection). Cells were cultured in DMEM medium supplemented with 10% FCS (Flow Laboratories, Inc.) in a 5% CO_2-water-saturated atmosphere. The GST-Grb2 fusion protein has been previously described (Ponzetto *et al.*, 1996). Antisera and monoclonal anti-*Met* antibodies were kindly provided by Dr M Prat. Monoclonal anti-p85 and anti-Myc antibodies were from Upstate Biotechnology (UBI) and Sigma, respectively.

Mutagenesis, cloning and expression of cDNA constructs

To generate the *Met* carboxy-terminal mutations an *in vitro* oligonucleotide site-directed mutagenesis system (Promega) was used as previously reported (Ponzetto *et al.*, 1993). *TPR–MET* cDNAs carrying the indicated mutations were reconstructed in the pMT2 vector, for transient expression in COS-1 cells. The plasmid (pSG5 p110CAAX) encoding the activated form of PI 3-kinase (p110*), obtained by addition of the C-terminal farnesylation signal from *Ha-Ras*, has been previously described (Khwaja *et al.*, 1997).

Plasmids were transfected in COS-1 and MDCK cells by the lipofection procedure (GIBCO–BRL). For selection of stable transfectants, cells (MDCK and Fisher rat fibroblasts) were cotransfected with the appropriate plasmid and with pSV2neo. Cells were maintained in DMEM containing 10% calf serum and 750 μg/ml G418.

Immunoprecipitation and Western blotting

Cells were lysed with EB buffer (100 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% glycerol, 1% Triton X-

100, 1 mM Sodium orthovanadate) and a cocktail of protease inhibitors (pepstatin, leupeptin, aprotinin and PMSF). Immunoprecipitation (with the indicated antibodies) was performed in EB buffer. After SDS–PAGE, proteins were transferred to Hybond-ECL membranes (Amersham). Filters were probed with the appropriate antibodies and specific binding was detected by the Enhanced Chemiluminescence System (ECL, Amersham).

In vitro *kinase assay*

Tpr–Met proteins were immunoprecipitated with anti-*Met* antibodies coupled to Protein A-Sepharose. Immunocomplexes were washed with EB buffer and incubated with kinase buffer (KB: 50 mM HEPES pH 7.5, 150 mM NaCl, 12.5 mM $MgCl_2$) in the presence of [γ-^{32}P]ATP. The reaction was carried out at 25°C for 15 min, and stopped by adding concentrated boiling Laemmli buffer. The eluted proteins were subjected to SDS–PAGE followed by autoradiography.

Focus forming assay

Transfection of the *TPR–MET* constructs in Fisher Rat fibroblasts was carried out using the DNA-calcium phosphate coprecipitation method (CellPhect Transfection Kit, Pharmacia). Cells (one 10 cm dish at 60% confluence) were transfected with 10 μg of DNA. After transfection cells were split 1 : 10 and kept in DMEM medium with 5% FCS. Formation of foci was detected in 2–3 weeks.

Experimental metastasis assays

Experimental metastasis assays were carried out in nude mice. Mice (6/each group) were injected in the tail vein with 10^6 transformed cells (pools of 10 foci). The experiment was repeated three times. Two weeks was chosen as a reference point to evaluate lethality (due to lung metastases) since in this series of experiments animals injected with cells transformed by *Tpr–Met*^{wt} were invariably all dead by this time. All animals injected with cells transformed by *Tpr–Met* signaling mutants would eventually die at later times. The presence of lung metastasis was evaluated at necropsy and confirmed by histopathological examination of the lungs.

Transwell invasion assays

Invasion assays were performed using Transwells (6.5 mm, Costar, Cambridge, MA, USA). The polycarbonate membranes (8-mm pore size) on the bottom of the upper compartment of the Transwells were coated with 1.2 mg/ml Matrigel (Collaborative Research, Waltham, MA, USA). Cells (10^5 in 200 μl of DMEM 5% FCS) were placed in the upper compartment. One ml of DMEM 5% FCS was added to the lower compartment. The Transwell containing plates were incubated at 37°C in a 5% CO_2 atmosphere saturated with H_2O for 48 h. At the end of incubation, cells that had dived to the lower compartment of the Transwell were fixed with 11% glutharaldehyde for 15 min at room temperature, washed three times with distilled water, and stained with 0.1% crystal violet-20% methanol for 20 min at room temperature for counting.

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